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(54) Title: VACCINE ADJUVANT

(57) Abstract

An immunogen/vaccine adjuvant composition containing an immunogen in an amount effective to stimulate an immune response and as a vaccine adjuvant a 1H-imidazo[4,5-c]quinolin-4-amine in an amount effective to increase the immune response to the immunogen.

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- 1 -

VACCINE ADJUVANT

Field of the Invention

This invention relates to compositions comprising
5 a vaccine and a vaccine adjuvant. In another aspect
this invention relates to vaccine adjuvants.

Description of the Related Art

In the field of immunology it has been well known
10 for many years that immune response to certain antigens
which are otherwise weakly immunogenic can be enhanced
through the use of vaccine adjuvants. Such adjuvants
potentiate the immune response to specific antigens and
are therefore the subject of considerable interest and
15 study within the medical community.

A wealth of knowledge concerning the complexity
and sophistication of immune regulation
("immunomodulation") has become available in the past
decade. Coupled with currently available biosynthetic
20 and recombinant DNA technology, this knowledge is
permitting development of vaccines possessing antigenic
epitopes that were previously impossible to produce.
For example, currently available vaccine candidates
include synthetic peptides mimicking streptococcal,
25 gonococcal, and malarial antigens. These purified
antigens are generally weak immunogens, however, that
require adjuvants in order to evoke protective
immunity. Unfortunately, however, as detailed below,
conventional vaccine adjuvants possess a number of
30 drawbacks which limit their overall use and
effectiveness.

Over the years, Freund's complete or incomplete
(i.e., without mycobacteria) adjuvants have been
considered the classic adjuvants to which most other
35 adjuvants are compared. However, clinical use of such
adjuvants in animals or humans is precluded because
they produce granulomas at the site of injection; fever

- 2 -

and other toxic effects; and tuberculin hypersensitivity. Other materials, such as mineral oil and aluminum hydroxide, have also been used as adjuvants, but they invariably suffer from 5 disadvantages. For example, mineral oil is known to produce tissue irritation and to be potentially oncogenic. Aluminum hydroxide, the only approved adjuvant in the United States, also induces granulomas at the inoculation site and furthermore it does not 10 effectively induce cell mediated immunity. Moreover, many of the adjuvants currently available have limited utility because they contain components which are not metabolizable in humans. Additionally, most adjuvants are difficult to prepare in that they may require time 15 consuming procedures and the use, in some cases, of elaborate and expensive equipment to formulate a vaccine and adjuvant system.

For a thorough discussion of various immunological adjuvants, see "Current Status of Immunological 20 Adjuvants", Ann. Rev. Immunol., 1986, 4, pp. 369-388. See also U.S. Pat. Nos. 4,806,352; 5,026,543; and 5,026,546 for disclosures of various vaccine adjuvants appearing in the patent literature.

In recent years, in an ongoing attempt to find new 25 adjuvants for vaccines which would overcome the drawbacks and deficiencies of conventional adjuvants, there have been those within the medical community who have postulated that the adjuvant potential of various substances can be directly correlated to their 30 immunomodulatory capabilities, i.e., the ability to affect the immune system in some fashion. For example, increased cytokine (e.g., TNF, IL-2, IL-6, IL-8, alpha-interferon, etc.) production by a particular substance could be interpreted as being indicative of a 35 beneficial effect if used as an adjuvant for vaccines. The latter, however, has not always been found to be true.

- 3 -

Staphylococcus enterotoxin B, for example, has not been found to be immunoenhancing for either cell-mediated (e.g., cytotoxic T-cell lymphocytes) or humoral immune responses (i.e., specific antibody production) even though the enterotoxin has been shown to increase the level of production of various cytokines such as IL-2, TNF, gamma-interferon, etc. (see, e.g., J. Immunol., 1975, 115, 575 (Smith et al.) and Infection and Immunity, 1978, 22, 62 (Lansford et al.)) The same situation has been shown to be true for Toxic Shock Syndrome toxin-1 and a variety of other substances as well (see, e.g., J. Infectious Diseases, 1986, 153, 722 (Poindexter et al.), Immunology, 1986, 58, 203 (Meusen et al.), and J. Clin. Invest., 1984, 73, 1312 (Ikejima et al.)).

In view of the foregoing, it can be readily seen that the general immunomodulatory effects of various substances is not necessarily an accurate barometer of their immunoenhancing capabilities. Accordingly, this fact has frustrated the search for materials which would be effective adjuvants for various vaccines and as a result such materials are constantly sought by and are in high demand within the medical community.

Clearly, an adjuvant formulation which elicits potent cell-mediated and humoral immune responses to a wide range of antigens in humans and domestic animals, but lacking the side effects of conventional adjuvants, such as Freund's complete adjuvant, would be highly desirable. It was against this background that Applicants began their search for an effective vaccine adjuvant.

Summary of the Invention

This invention provides an immunogen/vaccine adjuvant composition comprising an immunogen in an amount effective to stimulate an immune response and as a vaccine adjuvant a 1H-imidazo[4,5-c]quinolin-4-amine

- 4 -

in an amount effective to increase the immune response to the immunogen.

This invention also provides a method of increasing the immune response to an immunogen,

- 5 comprising the step of administering (i) the immunogen in an amount effective to stimulate an immune response, and (ii) as a vaccine adjuvant a 1H-imidazo[4,5-c]quinolin-4-amine in an amount effective to increase the immune response.

10 Certain 1H-imidazo[4,5-c]quinolin-4-amines have been disclosed as antiviral agents (see, e.g., U.S. Pat. Nos. 4,689,338 (Gerster) and 4,929,624 (Gerster et al.), European Patent Application 90.301776.3 (Gerster) and commonly assigned copending U.S. patent applications 07/838,475 (Gerster et al.), 07/754,610 (Gerster et al.), and 07/788,565 (Gerster et al.). Certain of these compounds are also known to induce biosynthesis of cytokines such as interferons, interleukins, and tumor necrosis factor in humans and 20 in mice. In this invention, however, the 1H-imidazo[4,5-c]quinolin-4-amine functions as a vaccine adjuvant (i.e., it is an immunostimulatory substance that potentiates humoral and/or cell mediated immune responses to an immunogen). These compounds are 25 relatively small synthetic organic molecules that are well characterized and substantially free of contaminants that can cause undesired effects. They are generally suitably nontoxic and do not cause undue irritation at the site of injection. Therefore this 30 invention avoids the shortcomings seen with some vaccine adjuvants of the prior art.

Detailed Description of the Invention

As used herein the term "immunogen/vaccine 35 adjuvant composition" refers to a combination of an immunogen and a 1H-imidazo[4,5-c]quinolin-4-amine, whether that combination is in the form of an admixture

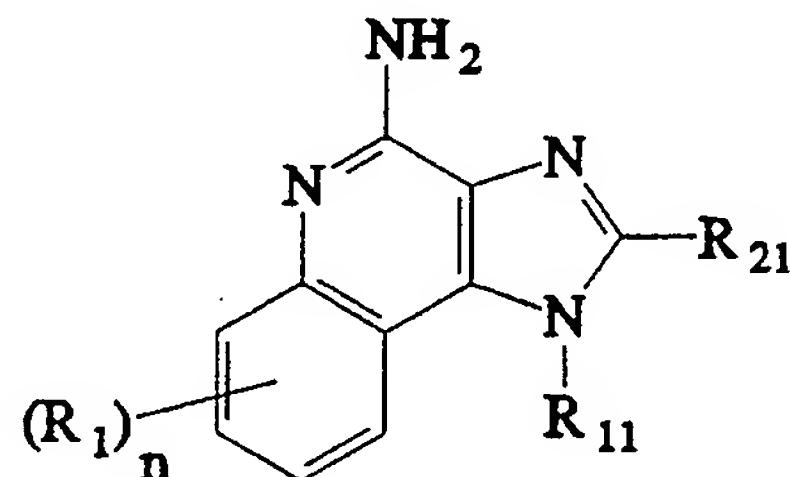
- 5 -

of the two components in a pharmaceutically acceptable carrier or in the form of separate, individual components, for example in the form of a kit comprising an immunogen as one component and the 1H-imidazo[4,5-c]quinolin-4-amine as another component.

The vaccine adjuvant component of a composition of the invention is a 1H-imidazo[4,5-c]quinolin-4-amine. It has been found that compounds of this class induce biosynthesis of a variety of cytokines in human and murine cells. While the particular profile of cytokine induction varies to some extent from compound to compound within this class, it is thought that the general profile of cytokine induction common to the compounds of the class is responsible for the vaccine adjuvant activity of the compounds. Also, some compounds of this class have been shown to be potent stimulants of β -lymphocytes and therefore capable of increasing humoral immune response.

Preferably the 1H-imidazo[4,5-c]quinolin-4-amine is a compound defined by one of Formulas I-V below:

25



30

I

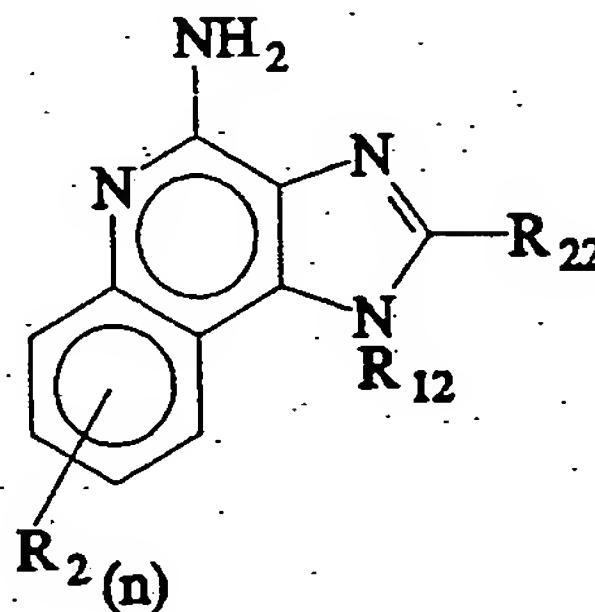
wherein

R₁₁ is selected from the group consisting of alkyl, hydroxyalkyl, acyloxyalkyl, benzyl, (phenyl)ethyl and phenyl, said benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by one or two moieties independently selected from

- 6 -

the group consisting of alkyl of one to about four carbon atoms, alkoxy of one to about four carbon atoms and halogen, with the proviso that if said benzene ring is substituted by two of said moieties, then said 5 moieties together contain no more than 6 carbon atoms; R₂₁ is selected from the group consisting of hydrogen, alkyl of one to about eight carbon atoms, benzyl, (phenyl)ethyl and phenyl, the benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the 10 benzene ring by one or two moieties independently selected from the group consisting of alkyl of one to about four carbon atoms, alkoxy of one to about four carbon atoms and halogen, with the proviso that when the benzene ring is substituted by two of said 15 moieties, then the moieties together contain no more than 6 carbon atoms; and each R₁ is independently selected from the group consisting of alkoxy of one to about four carbon atoms, halogen and alkyl of one to about four carbon atoms, and n is an integer from 0 to 20 2, with the proviso that if n is 2, then said R₁ groups together contain no more than 6 carbon atoms;

25



30

II

wherein

35 R₁₂ is selected from the group consisting of straight chain or branched chain alkenyl containing 2

- 7 -

to about 10 carbon atoms and substituted straight chain or branched chain alkenyl containing 2 to about 10 carbon atoms, wherein the substituent is selected from the group consisting of straight chain or branched

5 chain alkyl containing 1 to about 4 carbon atoms and cycloalkyl containing 3 to about 6 carbon atoms; and cycloalkyl containing 3 to about 6 carbon atoms substituted by straight chain or branched chain alkyl containing 1 to about 4 carbon atoms; and

10 R_{22} is selected from the group consisting of hydrogen, straight chain or branched chain alkyl containing one to about eight carbon atoms, benzyl, (phenyl)ethyl and phenyl, the benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the

15 benzene ring by one or two moieties independently selected from the group consisting of straight chain or branched chain alkyl containing one to about four carbon atoms, straight chain or branched chain alkoxy containing one to about four carbon atoms, and halogen,

20 with the proviso that when the benzene ring is substituted by two such moieties, then the moieties together contain no more than 6 carbon atoms; and

each R_2 is independently selected from the group consisting of straight chain or branched chain alkoxy

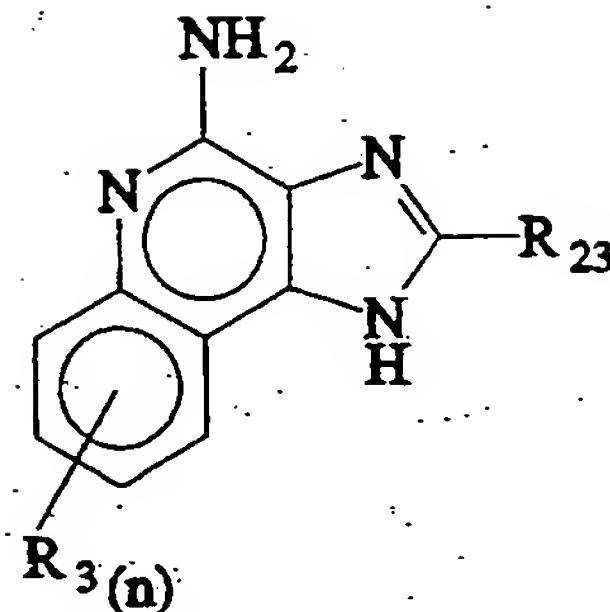
25 containing one to about four carbon atoms, halogen, and straight chain or branched chain alkyl containing one to about four carbon atoms, and n is an integer from zero to 2, with the proviso that if n is 2, then said R_2 groups together contain no more than 6 carbon atoms;

30

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- 8 -

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III

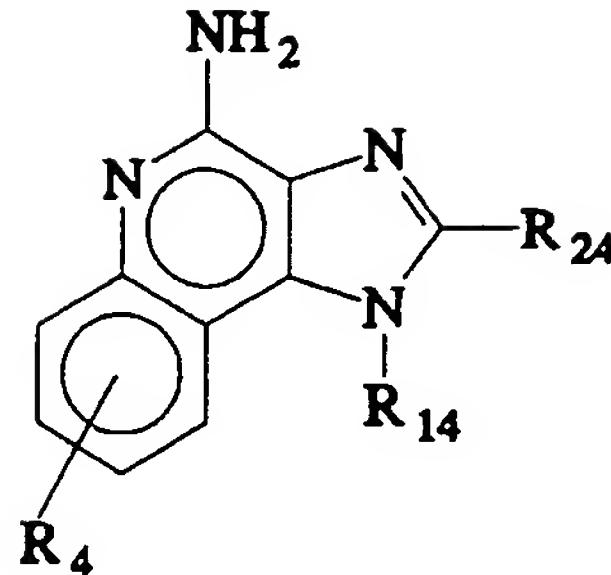
wherein

R_{23} is selected from the group consisting of
 15 hydrogen, straight chain or branched chain alkyl of one
 to about eight carbon atoms, benzyl, (phenyl)ethyl and
 phenyl, the benzyl, (phenyl)ethyl or phenyl substituent
 being optionally substituted on the benzene ring by one
 or two moieties independently selected from the group
 20 consisting of straight chain or branched chain alkyl of
 one to about four carbon atoms, straight chain or
 branched chain alkoxy of one to about four carbon
 atoms, and halogen, with the proviso that when the
 benzene ring is substituted by two such moieties, then
 25 the moieties together contain no more than 6 carbon
 atoms; and

each R_3 is independently selected from the group
 consisting of straight chain or branched chain alkoxy
 of one to about four carbon atoms, halogen, and
 30 straight chain or branched chain alkyl of one to about
 four carbon atoms, and n is an integer from zero to 2,
 with the proviso that if n is 2, then said R_3 groups
 together contain no more than 6 carbon atoms;

- 9 -

5



10

IV

wherein R_{14} is $-\text{CHR}_A\text{R}_B$

wherein

- 15 R_B is hydrogen or a carbon-carbon bond, with the proviso that when R_B is hydrogen R_A is alkoxy of one to about four carbon atoms, hydroxyalkoxy of one to about four carbon atoms, 1-alkynyl of two to about ten carbon atoms, tetrahydropyranyl, alkoxyalkyl wherein the
- 20 alkoxy moiety contains one to about four carbon atoms and the alkyl moiety contains one to about four carbon atoms, 2-, 3-, or 4-pyridyl, and with the further proviso that when R_B is a carbon-carbon bond R_B and R_A together form a tetrahydrofuryl group optionally
- 25 substituted with one or more substituents independently selected from the group consisting of hydroxy and hydroxyalkyl of one to about four carbon atoms;

R_{24} is selected from the group consisting of hydrogen, alkyl of one to about four carbon atoms, phenyl, and substituted phenyl wherein the substituent is selected from the group consisting of alkyl of one to about four carbon atoms, alkoxy of one to about four carbon atoms, and halogen; and

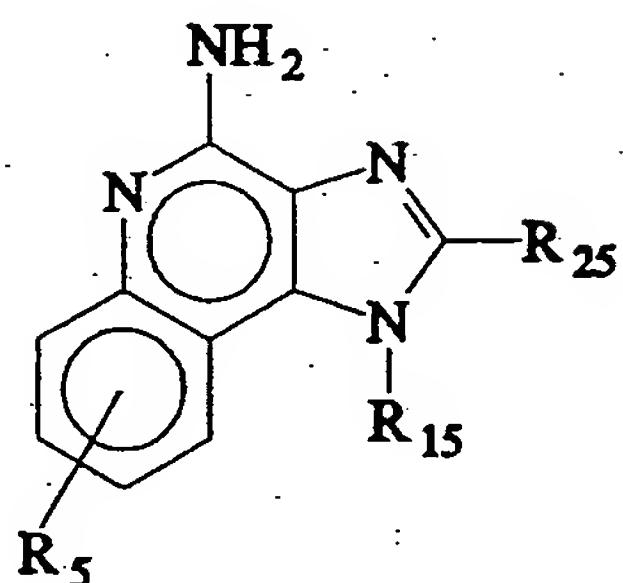
R_4 is selected from the group consisting of hydrogen, straight chain or branched chain alkoxy containing one to about four carbon atoms, halogen, and

- 10 -

straight chain or branched chain alkyl containing one to about four carbon atoms;

5

10



V

15 wherein

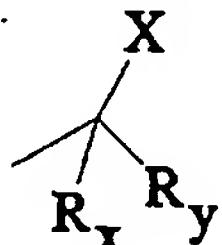
R₁₅ is selected from the group consisting of:
 hydrogen; straight chain or branched chain alkyl containing one to about ten carbon atoms and substituted straight chain or branched chain alkyl
 20 containing one to about ten carbon atoms, wherein the substituent is selected from the group consisting of cycloalkyl containing three to about six carbon atoms and cycloalkyl containing three to about six carbon atoms substituted by straight chain or branched chain
 25 alkyl containing one to about four carbon atoms; straight chain or branched chain alkenyl containing two to about ten carbon atoms and substituted straight chain or branched chain alkenyl containing two to about ten carbon atoms, wherein the substituent is selected
 30 from the group consisting of cycloalkyl containing three to about six carbon atoms and cycloalkyl containing three to about six carbon atoms substituted by straight chain or branched chain alkyl containing one to about four carbon atoms; hydroxyalkyl of one to
 35 about six carbon atoms; alkoxyalkyl wherein the alkoxy moiety contains one to about four carbon atoms and the alkyl moiety contains one to about six carbon atoms;

- 11 -

- acyloxyalkyl wherein the acyloxy moiety is alkanoyloxy of two to about four carbon atoms or benzoyloxy, and the alkyl moiety contains one to about six carbon atoms; benzyl; (phenyl)ethyl; and phenyl; said benzyl,
- 5 (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by one or two moieties independently selected from the group consisting of alkyl of one to about four carbon atoms, alkoxy of one to about four carbon atoms, and halogen, with the
- 10 proviso that when said benzene ring is substituted by two of said moieties, then the moieties together contain no more than six carbon atoms;

R₂₅ is

15



20 wherein

- R_x and R_y are independently selected from the group consisting of hydrogen, alkyl of one to about four carbon atoms, phenyl, and substituted phenyl wherein the substituent is selected from the group consisting of
- 25 alkyl of one to about four carbon atoms, alkoxy of one to about four carbon atoms, and halogen;

X is selected from the group consisting of alkoxy containing one to about four carbon atoms, alkoxyalkyl wherein the alkoxy moiety contains one to about four carbon atoms and the alkyl moiety contains one to about four carbon atoms, haloalkyl of one to about four carbon atoms, alkylamido wherein the alkyl group contains one to about four carbon atoms, amino, substituted amino wherein the substituent is alkyl or

- 30 carbon atoms and the alkyl moiety contains one to about four carbon atoms, haloalkyl of one to about four carbon atoms, alkylamido wherein the alkyl group contains one to about four carbon atoms, amino,
- 35 hydroxyalkyl of one to about four carbon atoms, azido, alkylthio of one to about four carbon atoms; and

- 12 -

R₅ is selected from the group consisting of hydrogen, straight chain or branched chain alkoxy containing one to about four carbon atoms, halogen, and straight chain or branched chain alkyl containing one 5 to about four carbon atoms;

or a pharmaceutically acceptable salt of any of the foregoing.

The compounds recited above are disclosed and claimed in the several patents and applications noted 10 above in the Summary of the Invention.

In instances where n can be zero, one, or two, n is preferably zero or one.

The substituents R₁-R₅ above are generally designated "benzo substituents" herein. The preferred 15 benzo substituent is hydrogen.

The substituents R₁₁-R₁₅ above are generally designated "1-substituents" herein. The preferred 1- substituent is 2-methylpropyl or 2-hydroxy-2-methylpropyl.

20 The substituents R₂₁-R₂₅ above are generally designated "2-substituents" herein. The preferred 2- substituents are hydrogen, alkyl of one to about six carbon atoms, alkoxyalkyl wherein the alkoxy moiety contains one to about four carbon atoms and the alkyl 25 moiety contains one to about four carbon atoms. Most preferably the 2-substituent is hydrogen, methyl, or ethoxymethyl.

Preferred compounds 1H-imidazo[4,5-c] quinolin-4- amines include:

30 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4- amine;

1-(2-hydroxy-2-methylpropyl)-1H-imidazo[4,5- c]quinolin-4-amine; and

1-(2-hydroxy-2-methylpropyl)-2-methyl-1H- 35 imidazo[4,5-c]quinolin-4-amine; and

- 13 -

1-(2-hydroxy-2-methylpropyl)-2-ethoxymethyl-1H-imidazo[4,5-c]quinolin-4-amine.

The 1H-imidazo[4,5-c]quinolin-4-amine is present (or administered, as appropriate to the form of the

- 5 immunogen/vaccine adjuvant composition) in an amount effective to increase the immune response to a particular immunogen. For example, in instances where the compound is administered independent of the immunogen, e.g., by separate injection, the compound is
- 10 generally administered in an amount of about 0.003 to about 5 mg/kg. The particular amount that constitutes an effective amount, however, depends to some extent upon certain factors, including the particular 1H-imidazo[4,5-c]quinolin-4-amine, the particular
- 15 immunogen being administered and the amount thereof, the immune response that is to be enhanced (humoral or cell mediated), the state of the immune system (e.g., suppressed, compromised, stimulated), the method and order of administration of the compound and the
- 20 immunogen, the species, and the desired therapeutic result. Accordingly it is not practical to set forth generally the amount that constitutes an effective amount of the 1H-imidazo[4,5-c]quinolin-4-amine. Those of ordinary skill in the art, however, can readily
- 25 determine the appropriate amount with due consideration of such factors.

As shown in the Examples that follow, a 1H-imidazo[4,5-c]quinolin-4-amine has the effect of enhancing both humoral and cell mediated immune response. Therefore the immunogen can be any material that raises either humoral or cell mediated immune response, or both. Suitable immunogens include live viral and bacterial immunogens and inactivated viral, tumor-derived, protozoal, organism-derived, fungal, and bacterial immunogens, toxoids, toxins, polysaccharides, proteins, glycoproteins, peptides, and the like. Conventional vaccine preparations, such as those used

- 14 -

in connection with BCG (live bacteria), cholera, plague, and typhoid (killed bacteria), hepatitis B, influenza, inactivated polio, and rabies (inactivated virus), measles, mumps, rubella, oral polio, and yellow fever (live virus), tetanus and diphtheria (toxoids), hemophilus influenzae b, meningococcal, and pneumococcal (bacterial polysaccharides) can be used as the immunogen. Because the 1H-imidazo[4,5-c]quinolin-4-amine compounds induce biosynthesis of antiviral cytokines, in the instance of a live viral immunogen it is preferred to administer the virus prior to administration of the adjuvant compound in order that the viral infection can be established.

Furthermore, it is contemplated that certain currently experimental immunogens, especially materials such as recombinant proteins, glycoproteins, and peptides that do not raise a strong immune response, will also find use in connection with a 1H-imidazo[4,5-c]quinolin-4-amine. Exemplary experimental subunit immunogens include those related to viral disease such as adenovirus, AIDS, chicken pox, cytomegalovirus, dengue, feline leukemia, fowl plague, hepatitis A, hepatitis B, HSV-1, HSV-2, hog cholera, influenza A, influenza B, Japanese encephalitis, measles, parainfluenza, rabies, respiratory syncytial virus, rotavirus, wart, and yellow fever.

Preferred immunogens for use in this invention include T-dependent immunogens such as viral pathogens and tumor-derived immunogens.

A particular preferred immunogen for use in this invention is a herpes simplex II (HSV-2) glycoprotein subunit preparation prepared as described in J. Infect. Dis. 1987, 155, 914 (Stanberry et al.).

In the method of the invention, the immunogen is administered in an amount effective to stimulate an immune response. The amount that constitutes an effective amount depends to some extent upon certain

- 15 -

factors, including the particular immunogen, the particular adjuvant being administered and the amount thereof, the immune response that is to be enhanced (humoral or cell mediated), the state of the immune system (e.g., suppressed, compromised, stimulated), the method and order of administration of the compound and the immunogen, and the desired therapeutic result.

Accordingly it is not practical to set forth generally the amount that constitutes an effective amount of immunogen. Those of ordinary skill in the art, however, can readily determine the appropriate amount with due consideration of such factors.

The immunogen/vaccine adjuvant compositions of the invention can contain further pharmaceutically acceptable ingredients, excipients, carriers, and the like well known to those skilled in the art.

The immunogen/vaccine adjuvant composition of the invention can be administered to animals, e.g., mammals (human and non-human), fowl, and the like according to conventional methods well known to those skilled in the art (e.g., orally, subcutaneously, nasally, topically). It is preferred to administer the 1H-imidazo[4,5-c]quinolin-4-amine simultaneously with the immunogen (together in admixture or separately, e.g., orally or by separate injection) or subsequent to challenge with the immunogen. As seen in the Examples that follow (and as is common in the art) administration of the vaccine adjuvant prior to challenge with the immunogen can result in immunosuppression rather than stimulation.

The following Examples are provided to illustrate the invention.

In the Examples, "Compound A" designates 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine.

"Compound B" designates 1-(2-hydroxy-2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine. "Compound C" designates 1-(2-hydroxy-2-methylpropyl)-2-methyl-1H-

- 16 -

imidazo[4,5-c]quinolin-4-amine. "Compound D" designates 1-(2-hydroxy-2-methylpropyl)-2-ethoxymethyl-1H-imidazo[4,5-c]quinolin-4-amine.

5 STIMULATION OF ^3H -THYMIDINE UPTAKE IN CULTURES
OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

The test method described below demonstrates the ability of compounds to stimulate the uptake of ^3H -thymidine in human cells. Increased uptake of ^3H -thymidine indicates that the cells are actively dividing.

Blood Cell Preparation for Culture

Whole blood is collected by venipuncture into 15 heparin vacutainer tubes. Peripheral blood mononuclear cells (PBMC) are isolated using Ficoll-Paque® solution (available from Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The PBMC are washed with Hank's Balanced Salts Solution then diluted with RPMI 1640 20 medium containing 2.0 Mm L-glutamine, 10% fetal calf serum and 1% penicillin/streptomycin to obtain a concentration of 2×10^6 cells/mL.

Compound preparation

25 The compounds are dissolved in water then diluted with the medium used above to give the desired concentration.

Incubation

30 A 0.1 mL portion of compound solution is added to the wells (3 wells for each treatment) of a 96 well round bottom tissue culture plate. Control wells receive 0.1 mL portions of medium. A 0.1 mL portion of cell suspension (1×10^5 cells) is added to each well 35 and the plates are incubated for 48 hours at 37°C in the presence of 5% carbon dioxide. During the last 4 to

- 17 -

6 hour of culture 1 μ Ci of ^3H -thymidine (having a specific activity of 6.7 Ci/mmmole; available from New England Nuclear) is added to each well.

5 ^3H -Thymidine Uptake Measurement/Analysis

- Cultures are harvested and collected on glass fiber filter strips. Each strip is placed in a scintillation vial. A 1 to 2 mL portion of Aquasol®-2 Universal LSC Cocktail (available from DuPont) is added 10 to each well. After 15 minutes the radioactivity is counted for 1 minute in a scintillation counter. A stimulation index (SI) is calculated by dividing the counts per minute from the treatment wells by the counts per minute from the control wells.
- 15 Results are shown in the table below. Concentrations are the final concentrations found in the well after the addition of the cell suspension. The CPM value is the mean CPM of the three wells for each treatment. Phytohemagglutinin (PHA) and 20 lipopolysaccharide (LPS) are included as reference agents.

STIMULATION OF ^3H -THYMIDINE UPTAKE IN
CULTURES OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

TREATMENT	CPM \pm SEM	SI
Medium	4,859 \pm 392	1.0
PHA (5 $\mu\text{g}/\text{mL}$)	59,818 \pm 2,867	12.3
LPS (2 $\mu\text{g}/\text{mL}$)	3,228 \pm 433	0.7
Compound C (4 $\mu\text{g}/\text{mL}$)	30,119 \pm 636	6.2
Compound C (1 $\mu\text{g}/\text{mL}$)	29,596 \pm 3,221	6.1
Compound C (0.25 $\mu\text{g}/\text{mL}$)	43,055 \pm 9,383	8.9
Compound C (0.06 $\mu\text{g}/\text{mL}$)	24,336 \pm 2,756	5.0

- 18 -

STIMULATION OF ^3H -THYMIDINE UPTAKE
BY MURINE SPLEEN CELLS

The test method described below demonstrates the ability of compounds to stimulate the uptake of ^3H -thymidine by murine spleen cells. Increased uptake of ^3H -thymidine indicates that the cells are actively dividing.

Spleen Cell Preparation for Culture

- 10 Spleens are aseptically removed from male CFW mice 4 to 8 weeks of age and placed in 10 mL of Hank's Balanced Salts Solution (HBSS). A scalpel is used to remove the cells from the capsule. A single cell suspension is prepared by pipetting the suspension
- 15 several times using a 5.0 mL syringe equipped with a 19 gauge needle. The suspension is transferred to a 15 mL centrifuge tube and allowed to stand on ice for 4 minutes. The supernatant is removed with a 10 mL pipet, transferred to a clean 15 mL centrifuge tube and
- 20 centrifuged at 1200 rpm for 5 to 10 minutes. The supernatant is discarded. To remove the red blood cells, the pellet is resuspended in 5 mL of 0.15M ammonium chloride, let stand at room temperature for 5 minutes and then centrifuged at 1200 rpm for 5 to 10
- 25 minutes. The supernatant is discarded. The pellet is twice resuspended in 10 mL HBSS then centrifuged at 1200 rpm for 5 to 10 minutes. The supernatant is discarded. The pellet is resuspended in RPMI 1640 medium containing 2.0 mM L-glutamine, 10% fetal calf
- 30 serum, 1% penicillin/streptomycin and $5 \times 10^{-5}\text{M}$ 2-mercaptoethanol. The cells are counted then diluted with medium to give a concentration of 2×10^6 cells/mL.

Compound Preparation

- 35 The compounds are dissolved in water then diluted with medium to give the desired concentration.

- 19 -

Incubation

The same procedures and conditions as described above for uptake in PBMC are used.

5 ³H-Thymidine Uptake Measurement/Analysis

The same procedures and methods as described above for uptake in PBMC are used.

Results are shown in the table below.

Concentrations are the final concentrations found in

10. the well after the addition of the cell suspension. The CPM value is the mean CPM of the three wells for each treatment. Concanavalin A (ConA), lipopolysaccharide (LPS), staphylococcal enterotoxin B (SEB) and polyriboinosinic acid-polyribocytidylic acid (Poly IC)
15 are included as reference agents.

- 20 -

STIMULATION OF ^3H -THYMIDINE UPTAKE
BY MURINE SPLEEN CELLS

	TREATMENT	CPM	SI
	Medium	13,728	1.0
5	ConA (5 $\mu\text{g/mL}$)	488,180	35.6
	LPS (5 $\mu\text{g/mL}$)	114,023	8.3
	SEB (1 $\mu\text{g/mL}$)	303,213	24.2
	Poly IC (5 $\mu\text{g/mL}$)	36,102	2.6
	<u>Compound</u>		
10	C (1 $\mu\text{g/mL}$)	161,573	11.8
	C (0.1 $\mu\text{g/mL}$)	147,356	10.7
	C (0.01 $\mu\text{g/mL}$)	67,960	5.0
	C (0.001 $\mu\text{g/mL}$)	20,004	1.4
15	C (0.0001 $\mu\text{g/mL}$)	17,759	1.3
	A (1 $\mu\text{g/mL}$)	149,940	10.9
	A (0.1 $\mu\text{g/mL}$)	87,753	6.4
	A (0.01 $\mu\text{g/mL}$)	21,188	1.5
	A (0.001 $\mu\text{g/mL}$)	21,270	1.5
20	B (1 $\mu\text{g/mL}$)	146,980	10.7
	B (0.1 $\mu\text{g/mL}$)	51,880	3.8
	B (0.01 $\mu\text{g/mL}$)	19,525	1.4
	B (0.001 $\mu\text{g/mL}$)	20,596	1.5
	B (0.0001 $\mu\text{g/mL}$)	22,076	1.6
25	D (1 $\mu\text{g/mL}$)	174,203	12.7
	D (0.1 $\mu\text{g/mL}$)	165,630	12.1
	D (0.01 $\mu\text{g/mL}$)	180,606	13.2
	D (0.001 $\mu\text{g/mL}$)	116,380	8.5
	D (0.0001 $\mu\text{g/mL}$)	25,689	1.9

- 21 -

STIMULATION OF ANTIBODY PRODUCTION
IN MURINE SPLEEN CELLS

The test method described below demonstrates the ability of compounds to stimulate antibody production 5 in murine spleen cells.

Spleen Cell Preparation for Culture

The spleen cells are prepared as described above except that they are diluted in 6 well tissue culture 10 plates to give a final concentration of 1×10^7 cells/mL.

Compound Preparation

The compounds are dissolved in water then diluted 15 with medium to give the desired concentration.

Incubation

A 0.1 mL portion of compound solution is added to each well (2 wells for each treatment). Control wells 20 receive medium. The final volume in the well is adjusted to 1 mL with medium. The plates are incubated for 72 hours at 37°C in the presence of 5% carbon dioxide.

25 Antibody Production Measurement/Analysis

Antibody production is measured by utilizing a modified Jerne Plaque Assay. Briefly stated, the method is as follows. Plastic culture dishes are coated with 2 mL of poly-L-lysine (50 $\mu\text{g}/\text{mL}$). After 15 minutes the 30 plates are washed with phosphate buffered saline (PBS) and 2 mL of washed sheep red blood cells (SRBC) diluted 1:20 in PBS is added. After 15 minutes the plates are swirled, allowed to settle for another 15 minutes and rinsed with buffered saline. Finally, 1.5 mL of 35 phosphate-buffered saline, pH 7.2, is added to each plate along with 2.5×10^5 spleen cells. The plates are

- 22 -

then incubated in the presence of guinea pig complement at 37°C for 1 hour, after which plaque forming cells (PFC) are counted under slight magnification. Results are presented as the mean PFC/culture \pm SEM (standard error of the mean). A stimulation index (SI) is calculated by dividing the PFC from the treatment wells by the PFC from the control (medium) wells.

Results are shown in the table below.

Concentrations are the final concentrations found in the well after both the cell suspension and the compound solution have been added. The PFC value is the mean PFC of the 2 wells for each treatment group. Lipopolysaccharide (LPS) and polyriboinosinic acid-polyribocytidylic acid (PIC) are included as reference agents.

- 23 -

STIMULATION OF ANTIBODY PRODUCTION IN MURINE SPLEEN CELLS				
	TREATMENT	PFC/Culture	SI	PFC/ 10^6 cells
	Medium	167±18	1.0	30
5	LPS (10 μ g/mL)	1,555±208	9.3	179
	LPS (3 μ g/mL)	1,300±391	7.8	118
	LPS (1 μ g/mL)	1,150±232	6.9	153
	PIC (10 μ g/mL)	604±227	3.6	106
	PIC (3 μ g/mL)	365±142	2.2	49
10	PIC (1 μ g/mL)	273±15	1.6	29
	<u>Compound</u>			
	C (10 μ g/mL)	1,419±219	8.5	121
	C (3 μ g/mL)	1,271±67	7.6	190
15	C (1 μ g/mL)	1,465±311	8.8	274

STIMULATION OF B CELLS
IN MURINE SPLEEN CELLS

20 The test method described below demonstrates the ability of compounds to stimulate B cells in murine spleen cells.

Spleen Cell Preparation for Culture

25 Spleen cells are prepared as described above in connection with the uptake of 3 H-thymidine.

Compound Preparation

The compounds are dissolved in water then diluted 30 with medium to give the desired concentration.

- 24 -

Incubation

A 0.9 mL portion of cell suspension is added to each well of a 12 well tissue culture plate. A 0.1 mL portion of compound solution is added to the wells (2 5 wells for each treatment). Control wells receive 0.1 mL portions of medium. The plates are incubated for 72 hours at 37°C in the presence of 5% carbon dioxide.

Quantitation of B and T Cells

10 The cell culture is removed from the well, combined with the culture from the second well, and washed twice with Hanks Balanced Salts Solution. The cells are diluted with phosphate buffered saline (PBS) supplemented with 1% fetal calf serum (FCS) to give a 15 concentration of 1×10^6 cells/100 μ L. The cells are stained with antibody for 30 minutes at 4°C.

Fluorescein isothiocyanate labeled goat anti-mouse immunoglobulin antibody (FITC α Ig) functions as the B cell marker. Fluorescein isothiocyanate labeled anti 20 mouse Thy 1.2 antibody functions as the T cell marker. The cells are then washed twice with PBS supplemented with 1% FCS then analyzed for fluorescence using a Becton Dickinson FACSCAN. The results are reported as the percentage of the total cells, both the whole 25 (unseparated) cells and the blast-like cells, that are positive for the marker.

The results are shown in the table below. The concentrations are the final concentrations in the well after both the cell suspension and the compound 30 solution have been added. Lipopolysaccharide is included as a reference agent.

- 25 -

QUANTITATION OF B AND T CELLS IN MURINE SPLEEN CELL CULTURES					
		FITC α Ig		FITC Anti Thy 1.2	
	TREATMENT	Whole	Blast	Whole	Blast
5	Medium	56.5	-	34.7	-
	LPS (5 µg/mL)	73.1	93.6	15.5	9.4
	<u>Compound</u>				
10	C (4 µg/mL)	72.3	97.1	14.1	10.7
	C (1 µg/mL)	75.0	97.0	14.1	7.3
	C (0.25 µg/mL)	74.0	96.0	12.8	9.9

ENHANCEMENT OF ANTIBODY FORMATION IN MICE

15 The test method described below demonstrates the ability of compounds to enhance antibody formation in mice to sheep red blood cells (a T-dependent antigen). On day 0, male CFW mice 4 to 8 weeks of age are injected intraperitoneally with sheep red blood cells 20 (1 X 10⁷ in phosphate buffered saline). Also on day 0, test compounds are dissolved in sterile water then injected intraperitoneally (3 mice for each treatment). On day 4 the mice are sacrificed and the spleens are removed. Single cell suspensions are prepared in 25 phosphate buffered saline to give a final concentration of 5 X 10⁵ cells/mL for use in a modified Jerne Plaque Assay. The assay is performed as described above in connection with antibody formation in spleen cell cultures. The results are reported as plaque forming 30 cells (PFC) per 10⁶ cells and per spleen. A stimulation index (SI) is calculated by dividing the PFC value for

- 26 -

the treatment group by the PFC value for the control (SRBC but no compound) group.

Results are shown in the table below. Values are the average number of plaque forming cells (PFC) \pm SEM.

- 5 Each data point is the average of three mice pooled. Lipopolysaccharide (LPS) and polyriboinosinic acid-polyribocytidyllic acid (Poly IC) are included as reference agents.

10

ENHANCEMENT OF ANTIBODY PRODUCTION IN MICE

15

20

25

TREATMENT	PFC/ 10^6 CELLS	PFC/ SPLEEN	SI
Saline	1	11	
SRBC	7 \pm 1	473	1.0
LPS (1 mg/Kg) + SRBC	246 \pm 24	12,054	35.1
Poly IC (100 μ g/Kg) + SRBC	74 \pm 11	5,180	10.5
Compound C (10 mg/Kg) + SRBC	21 \pm 2	1,372	3.0
Compound C (3 mg/Kg) + SRBC	82 \pm 7	6,123	11.7
Compound C (1 mg/Kg) + SRBC	58 \pm 7	3,789	8.3

SUPPRESSION OF ANTIBODY FORMATION IN MICE

This test method is the same as the one described above for enhancement of antibody formation except that the compounds are administered on day minus 1 and 1 \times 30 10^8 SRBC are administered on day 0. The percent suppression is calculated as follows:

- 27 -

(PFC value of SRBC only - PFC value of treatment) x 100

PFC value of SRBC only

The results are shown in the table below.

5

SUPPRESSION OF ANTIBODY FORMATION IN MICE

10

TREATMENT	PFC/ 10 ⁶ CELLS	PFC/ SPLEEN	% SUPPRESS
Saline	1±1	38	-
SRBC	594±41	34,000	-
Compound C (1 mg/Kg) + SRBC	129±16	9,500	78.3
Compound C (3 mg/Kg) + SRBC	87±8	6,700	85.4
Compound C (1 mg/Kg) + saline day 0	3±1	220	-

15

The experiments set forth below illustrate the adjuvant effect in guinea pigs of 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine used in connection
20 with a herpes simplex 2 (HSV-2) glycoprotein subunit vaccine.

HSV-2 glycoprotein preparation

HSV-2 (strain MS) infected Vero cells were
25 solubilized and the glycoproteins were purified by lentil-lectin sepharose chromatography. The final preparation contained all three HSV-2 glycoproteins, gB, gD, and gG, that were evaluated. The glycoprotein preparation was diluted to contain 35 µg/0.1 mL total
30 glycoprotein. Glycoprotein administration is described below in connection with the experimental design.

Treatment Groups

1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine (one percent by weight in a cream containing water (76.5%), isosteric acid (10%), stearyl alcohol (3.1%), polysorbate 60 (2.55%), cetyl alcohol (2.2%), benzyl alcohol (2%), glycerin (2%), sorbitan monostearate (0.45%), methylparaben (0.2%), and propylparaben (0.02%) was administered to guinea pigs as described below intravaginally at a concentration of 5 mg/kg/day for 5 days beginning either simultaneously with glycoprotein administration ("S group"), or after a delay of 48h after glycoprotein administration ("D group"). The hydrochloride salt was administered in water subcutaneously at a dose of 3 mg/kg/day for 10 days beginning simultaneously with glycoprotein administration ("subQ S group"). Complete Freund's adjuvant ("CFA", Sigma) was administered as a 1:1 mixture of the adjuvant and the glycoprotein ("CFA Group"). An unimmunized infected control group was 15 maintained. Also one group was given the glycoprotein alone ("glycoprotein group").

Experimental design

Hartley female guinea pigs (Charles River Breeding Laboratory, Wilmington, Mass.) weighing 200-300 g were immunized with 35 µg of HSV-2 glycoproteins in the hind footpads, first 35 days prior to vaginal inoculation with HSV-2 and again 14 days prior to inoculation.

Animals were inoculated intravaginally with $10^{5.7}$ pfu of either 333 strain HSV-2 (first experiment) or MS strain (ATCC VR-540) HSV-2 (second experiment). Samples of vaginal secretions were then collected over the next 10 days and stored frozen at -70°C prior to assay on Vero cells for viral concentration. During the acute 35 infection period (days 1-14), animals were evaluated daily for genital skin disease which was quantitated on a scale of 0-4 as described in J. Infect. Dis., 1982,

- 29 -

146, 397 (Stanberry et al.). Total lesion scores are the sum of these scores for days 1 through 14. After recovery from the acute infection, animals were examined daily from day 15-60 for evidence of recurrent 5 herpetic disease. Sera were collected from immunized animals just prior to intravaginal inoculation and again 14, 44, or 60 days later.

Enzyme-linked immunosorbent assay for HSV-2 antibodies

10 HSV-2 antibodies were quantified by an ELISA assay. Lectin purified HSV-2 glycoproteins were used as the solid phase and peroxidase-conjugated rabbit anti-guinea pig immunoglobulins (Accurate Chemical, Westbury, N.Y.) were used for detection of guinea pig 15 antibody. Absorbances were compared to a standardized control serum arbitrarily assigned a value of 10,000 ELISA units.

Statistics

20 Comparison of lesion scores for acute disease, viral shedding, and recurrent lesion days were done by two-tailed ANOVA with the Bonferroni correction to adjust for multiple groups. Data are expressed as mean ± S.E.

25

Acute Disease

To determine if 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine would increase the effectiveness of an HSV-2 glycoprotein vaccine, five treatment groups of 30 11 guinea pigs were used as follows:

- 1) unimmunized control group;
- 2) glycoprotein group;
- 3) D Group;
- 4) S Group; and
- 35 5) CFA Group.

Immunization with the HSV-2 glycoproteins alone significantly reduced the total lesion score from

- 30 -

19.1 ± 3.2 in the unimmunized control group to
3.9 ± 0.9 ($p < .001$). Because of the mild disease in the
glycoprotein group, no further significant reduction
could be demonstrated for the other groups, although
5 the total lesion score was less for each of the groups
receiving a vaccine adjuvant treatment. (D group,
2.8 ± 0.7; S Group, 2.2 ± 0.6; CFA Group, 1.2 ± 0.5).

10 Immunization with glycoprotein alone and also with
the several adjuvant preparations reduced vaginal viral
shedding compared to the unimmunized infected control
group.

Recurrent Disease

The recurrence pattern was similar for the
15 unimmunized control group and glycoprotein group
(4.9 ± 0.9 vs. 4.3 ± 0.9 recurrent lesion days,
respectively). The use of 1-(2-methylpropyl)-1H-
imidazo[4,5-c]quinolin-4-amine as an adjuvant, however,
significantly reduced recurrent lesion days to
20 0.8 ± 0.3 and 0.1 ± 0.1, respectively, for the S Group
and D Group ($p < .01$ for each compared to the
glycoprotein group). Only one of ten animals in the S
Group developed a recurrence, while eight of nine
recipients of glycoprotein alone ($p < .002$) developed a
25 recurrence. Three of ten animals in the CFA Group
developed recurrent lesions.

Antibody Response

Compared to the glycoprotein group, antibody
30 titers on the day of inoculation were marginally
increased in the S Group ($p < .05$), but increased by over
tenfold in the CFA Group ($p < .001$). Peak antibody titers
(day 44) in the unimmunized infected control group
approached the level induced in the glycoprotein group.
35 The CFA Group titers were higher than the unimmunized
control group and the groups receiving 1-(2-

- 31 -

methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine as a vaccine adjuvant.

The experiment described above was repeated, with
5 the addition of two treatment groups, in order to examine the effects of 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine given subcutaneously with glycoprotein ("SubQ S Group"), and the effects of 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine
10 alone ("Compound Group").

A pool of HSV-2 MS strain that had previously produced milder acute disease but more frequent recurrences was used in order to better observe effects on recurrent disease.

15

Acute Disease

The only groups to develop lesions acutely were the unimmunized groups (Compound Group, 9 of 9; unimmunized control group, 11 of 11) and the
20 glycoprotein group (6 of 11). Again, because of the significant effect of immunization with glycoprotein alone, only small adjuvant effects of 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine on the severity of the acute disease could be demonstrated
25 (differences in total lesion score ($p < .05$) for each compared to glycoprotein alone).

Vaginal viral shedding was also decreased by immunization with glycoprotein alone. The use of 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine as an
30 adjuvant, however, further decreased viral shedding. Compared to glycoprotein alone, viral shedding was decreased tenfold in the D Group, by another tenfold in the S Group ($p < .05$), and by yet another tenfold in the SubQ S Group ($p < .001$) on day one. Thus, there was
35 >99.9% reduction in the SubQ S Group compared to the glycoprotein group and a >99.9% reduction compared to the unimmunized control group. No virus was detected in

- 32 -

the CFA group. Treatment with 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine alone had no significant effect on vaginal viral shedding.

5 Recurrent Disease

Results are shown in the Table below:

EFFECT OF ADJUVANT ON THE PATTERN OF RECURRENT GENITAL HSV-2 DISEASE		
	Animals with recurrent lesions	No. days with herpetic lesions*
10	Group	
Unimmunized control	11/11	5.7 ± 0.8
Glycoprotein	9/11	2.5 ± 0.9 ^c
15 D Group	4/11	0.4 ± 0.2 ^b
S Group	3/11 ^b	0.3 ± 0.1 ^b
SubQ S Group	0/11 ^c	0 ^d
CFA Group	0/11 ^c	0 ^d
20 Compound Group	8/11	1.8 ± 0.5

* Mean ± SE per animal of days with recurrent herpetic lesions

^b P<.05 compared to Glycoprotein group

^c P<.001 compared to Glycoprotein group

^d P<.01 compared to Glycoprotein group

^e P<.001 compared to unimmunized control

30 Immunization with the glycoproteins alone significantly reduced recurrent lesion days compared to unimmunized controls (p<.01), but not the number of animals with recurrences. Compared to the glycoprotein alone, however, the use of 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine as an adjuvant further

- 33 -

significantly reduced recurrent lesion days and reduced the number of animals with recurrences. None of the animals in the SubQ S Group developed recurrences (p<.001 compared to glycoprotein alone). The Compound 5 Group also developed significantly fewer recurrences than the unimmunized control group (p<.001).

Antibody Response

Antibody titers in the CFA group were again over 10 tenfold higher than the glycoprotein group (p<.001) and the D Group, S Group, and SubQ S Group (p<.01). Groups that received 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine as an adjuvant did not, however, develop higher titers of HSV-2 antibody than the 15 glycoprotein group. The Compound Group developed higher antibody titers than the unimmunized control group (p<.05).

The results above indicate that 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine 20 augments the ability of the HSV-2 glycoprotein vaccine to decrease viral replication at the mucosal site, prevent clinical disease, and decrease the number of recurrences that develop after infection.

The most effective regimen involved subcutaneous 25 administration for 5 doses beginning at the time of immunization. The results were comparable to using CFA as an adjuvant. Animals that received intravaginal administration had decreased viral titers and fewer recurrent lesion days.

30 The addition of 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine to glycoprotein immunization had little effect on antibody titers but significantly increased the protection provided by the glycoprotein preparation especially against recurrent disease.

- 34 -

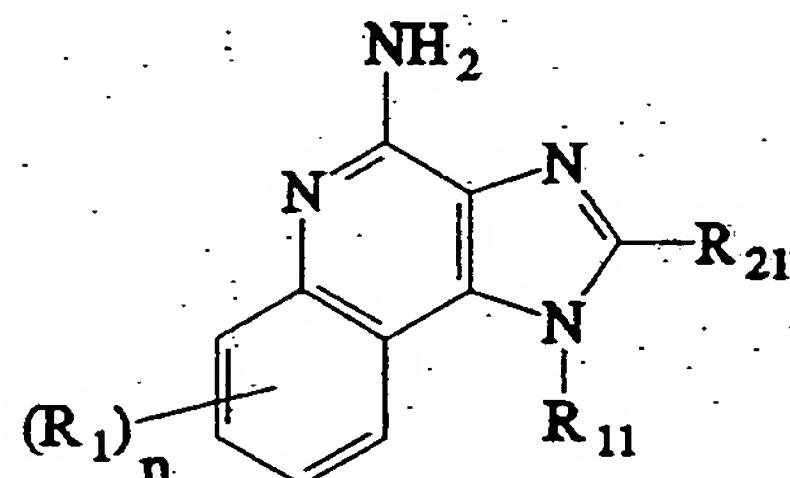
The claimed invention is:

1. An immunogen/vaccine adjuvant composition comprising an immunogen in an amount effective to stimulate an immune response and as a vaccine adjuvant a 1H-imidazo[4,5-c]quinolin-4-amine in an amount effective to increase the immune response to the immunogen.

- 10 2. An immunogen/vaccine adjuvant composition according to Claim 1, wherein the 1H-imidazo[4,5-c]quinolin-4-amine is a compound defined by one of Formulas I-V:

15

20



I

25 wherein

R₁₁ is selected from the group consisting of alkyl, hydroxyalkyl, acyloxyalkyl, benzyl, (phenyl)ethyl and phenyl, said benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by one or two moieties independently selected from the group consisting of alkyl of one to about four carbon atoms, alkoxy of one to about four carbon atoms, and halogen, with the proviso that if said benzene ring is substituted by two of said moieties, then said moieties together contain no more than 6 carbon atoms;

30 R₂₁ is selected from the group consisting of hydrogen,

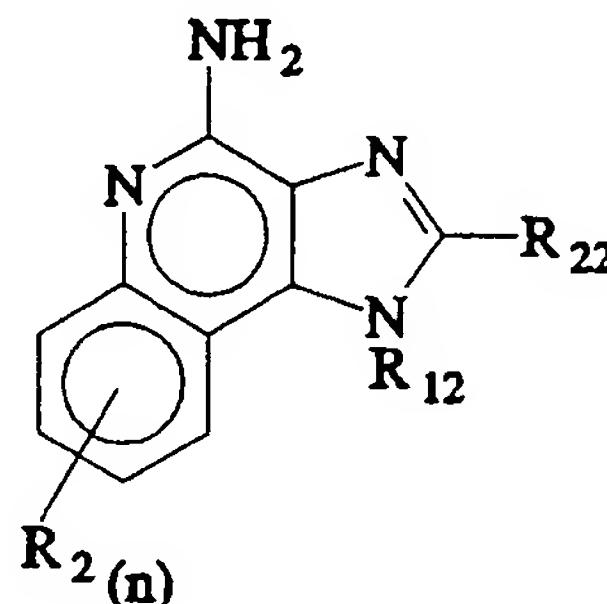
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- 35 -

alkyl of one to about eight carbon atoms, benzyl, (phenyl)ethyl, and phenyl, the benzyl (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by one or two moieties independently

- 5 selected from the group consisting of alkyl of one to about four carbon atoms, alkoxy of one to about four carbon atoms, and halogen, with the proviso that when the benzene ring is substituted by two of said moieties, then the moieties together contain no more
- 10 than 6 carbon atoms; and each R₁ is independently selected from the group consisting of alkoxy of one to about four carbon atoms, halogen, and alkyl of one to about four carbon atoms, and n is an integer from 0 to 2, with the proviso that if n is 2, then said R₁ groups
- 15 together contain no more than 6 carbon atoms;

20



25

II

wherein

- R₁₂ is selected from the group consisting of
- 30 straight chain or branched chain alkenyl containing 2 to about 10 carbon atoms and substituted straight chain or branched chain alkenyl containing 2 to about 10 carbon atoms, wherein the substituent is selected from the group consisting of straight chain or branched
- 35 chain alkyl containing 1 to about 4 carbon atoms and cycloalkyl containing 3 to about 6 carbon atoms; and

- 36 -

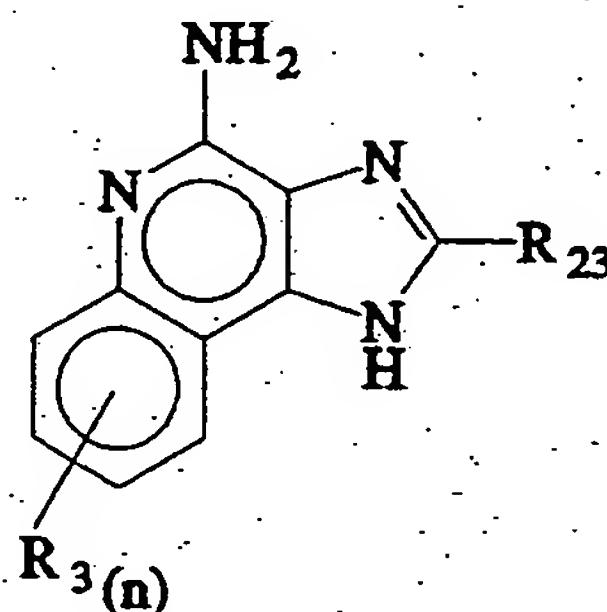
cycloalkyl containing 3 to about 6 carbon atoms substituted by straight chain or branched chain alkyl containing 1 to about 4 carbon atoms; and

R_{22} is selected from the group consisting of

- 5 hydrogen, straight chain or branched chain alkyl containing one to about eight carbon atoms, benzyl, (phenyl)ethyl and phenyl, the benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by one or two moieties independently
- 10 selected from the group consisting of straight chain or branched chain alkyl containing one to about four carbon atoms, straight chain or branched chain alkoxy containing one to about four carbon atoms, and halogen, with the proviso that when the benzene ring is
- 15 substituted by two such moieties, then the moieties together contain no more than 6 carbon atoms; and each R_2 is independently selected from the group consisting of straight chain or branched chain alkoxy containing one to about four carbon atoms, halogen, and
- 20 straight chain or branched chain alkyl containing one to about four carbon atoms, and n is an integer from zero to 2, with the proviso that if n is 2, then said R_2 groups together contain no more than 6 carbon atoms;

25

30.



III

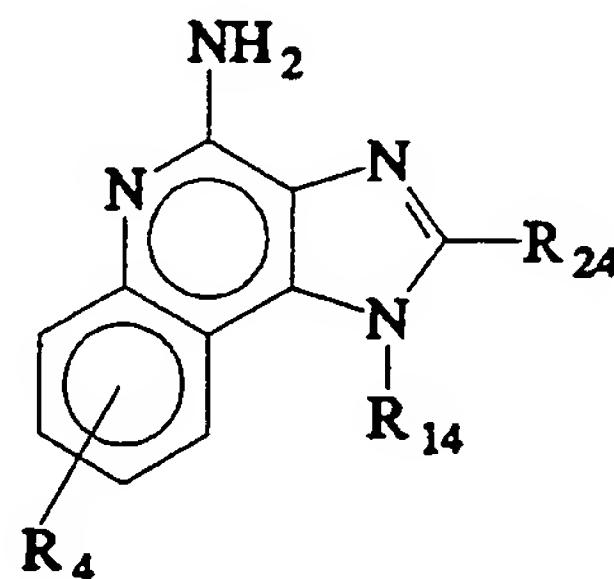
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- 37 -

wherein

- R_{23} is selected from the group consisting of hydrogen, straight chain or branched chain alkyl of one to about eight carbon atoms, benzyl, (phenyl)ethyl and phenyl, the benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by one or two moieties independently selected from the group consisting of straight chain or branched chain alkyl of one to about four carbon atoms, straight chain or branched chain alkoxy of one to about four carbon atoms, and halogen, with the proviso that when the benzene ring is substituted by two such moieties, then the moieties together contain no more than 6 carbon atoms; and
- 15 each R_3 is independently selected from the group consisting of straight chain or branched chain alkoxy of one to about four carbon atoms, halogen, and straight chain or branched chain alkyl of one to about four carbon atoms, and n is an integer from zero to 2,
- 20 with the proviso that if n is 2, then said R groups together contain no more than 6 carbon atoms;

25



30

IV

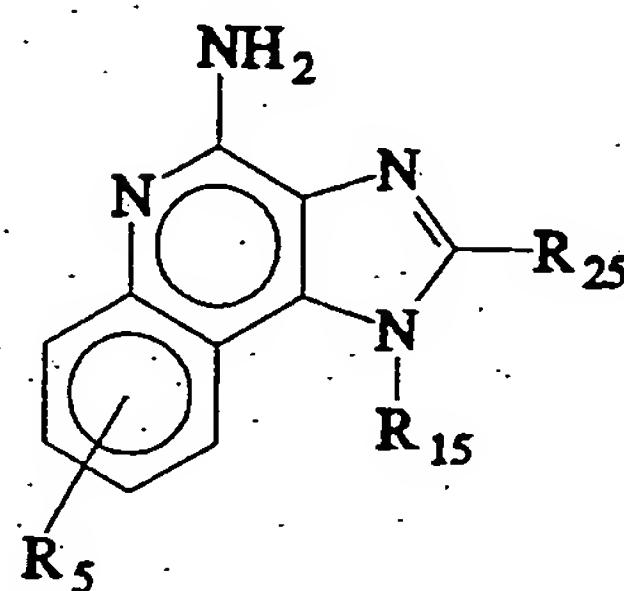
wherein R_{14} is $-CHR_AR_B$

- 38 -

wherein

- R_B is hydrogen or a carbon-carbon bond, with the proviso that when R_B is hydrogen R_A is alkoxy of one to about four carbon atoms, hydroxyalkoxy of one to about four carbon atoms, 1-alkynyl of two to about ten carbon atoms, tetrahydropyranyl, alkoxyalkyl wherein the alkoxy moiety contains one to about four carbon atoms and the alkyl moiety contains one to about four carbon atoms, 2-, 3-, or 4-pyridyl, and with the further proviso that when R_B is a carbon-carbon bond R_B and R_A together form a tetrahydrofuryl group optionally substituted with one or more substituents independently selected from the group consisting of hydroxy and hydroxyalkyl of one to about four carbon atoms;
- R_{24} is selected from the group consisting of hydrogen, alkyl of one to about four carbon atoms, phenyl, and substituted phenyl wherein the substituent is selected from the group consisting of alkyl of one to about four carbon atoms, alkoxy of one to about four carbon atoms, and halogen; and
- R_4 is selected from the group consisting of hydrogen, straight chain or branched chain alkoxy containing one to about four carbon atoms, halogen, and straight chain or branched chain alkyl containing one to about four carbon atoms;

30



35

wherein

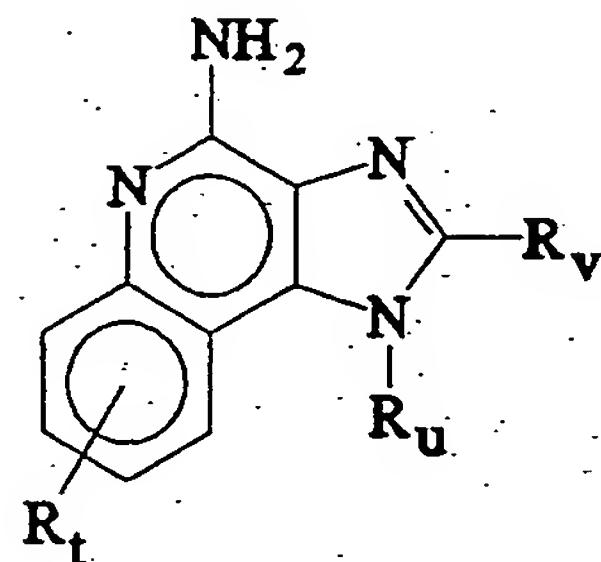
- R_{15} is selected from the group consisting of:
hydrogen; straight chain or branched chain alkyl
containing one to about ten carbon atoms and
5 substituted straight chain or branched chain alkyl
containing one to about ten carbon atoms, wherein the
substituent is selected from the group consisting of
cycloalkyl containing three to about six carbon atoms
and cycloalkyl containing three to about six carbon
10 atoms substituted by straight chain or branched chain
alkyl containing one to about four carbon atoms;
straight chain or branched chain alkenyl containing two
to about ten carbon atoms and substituted straight
chain or branched chain alkenyl containing two to about
15 ten carbon atoms, wherein the substituent is selected
from the group consisting of cycloalkyl containing
three to about six carbon atoms and cycloalkyl
containing three to about six carbon atoms substituted
by straight chain or branched chain alkyl containing
20 one to about four carbon atoms; hydroxyalkyl of one to
about six carbon atoms; alkoxyalkyl wherein the alkoxy
moiety contains one to about four carbon atoms and the
alkyl moiety contains one to about six carbon atoms;
acyloxyalkyl wherein the acyloxy moiety is alkanoyloxy
25 of two to about four carbon atoms or benzyloxy, and
the alkyl moiety contains one to about six carbon
atoms; benzyl; (phenyl)ethyl; and phenyl; said benzyl,
(phenyl)ethyl or phenyl substituent being optionally
substituted on the benzene ring by one or two moieties
30 independently selected from the group consisting of
alkyl of one to about four carbon atoms, alkoxy of one
to about four carbon atoms, and halogen, with the
proviso that when said benzene ring is substituted by
two of said moieties, then the moieties together
35 contain no more than six carbon atoms; or a
pharmaceutically acceptable salt thereof.

- 40 -

3. A composition according to Claim 1, wherein the 1H-imidazo[4,5-c] quinolin-4-amine is a compound of Formula VI:

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VI

15 wherein

R_t is selected from the group consisting of hydrogen, straight chain or branched chain alkoxy containing one to about four carbon atoms, halogen, and straight chain or branched chain alkyl containing one
20 to about four carbon atoms;

R_u is 2-methylpropyl or 2-hydroxy-2-methylpropyl;
and

R_v is hydrogen, alkyl of one to about six carbon atoms, or alkoxyalkyl wherein the alkoxy moiety
25 contains one to about four carbon atoms and the alkyl moiety contains one to about four carbon atoms.

4. A composition according to Claim 3, wherein R_t is hydrogen.

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5. A composition according to Claim 3, wherein R_t is hydrogen, R_u is 2-methylpropyl or 2-hydroxy-2-methylpropyl, and R_v is hydrogen, methyl, or ethoxymethyl.

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- 41 -

6. A composition according to Claim 1, wherein the compound is selected from the group consisting of 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine; 1-(2-hydroxy-2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine; 1-(2-hydroxy-2-methylpropyl)-2-methyl-1H-imidazo[4,5-c]quinolin-4-amine; and 1-(2-hydroxy-2-methylpropyl)-2-ethoxymethyl-1H-imidazo[4,5-c]quinolin-4-amine.
- 10 7. A composition according to Claim 1, wherein the immunogen is selected from the group consisting of a live viral immunogen, a live bacterial immunogen, an inactivated viral immunogen, an inactivated tumor-derived immunogen, an inactivated protozoal 15 immunogen, an inactivated organism-derived immunogen, an inactivated fungal immunogen, an inactivated bacterial immunogen, a toxoid, a toxin, a polysaccharide, a protein, a glycoprotein, and a peptide.
- 20 8. A composition according to Claim 1, wherein the immunogen is a conventional vaccine preparation.
9. A composition according to Claim 1, wherein 25 the immunogen is a recombinant subunit vaccine.
10. A composition according to Claim 1, wherein the immunogen is a T-dependent immunogen.
- 30 11. A composition according to Claim 1, wherein the immunogen is a herpes simplex 2 immunogen.
12. A composition according to Claim 1, wherein 35 the immunogen is herpes simplex 2 glycoprotein subunit preparation.

- 42 -

13. A composition according to Claim 1, comprising an admixture of the 1H-imidazo[4,5-c]quinolin-4-amine and the immunogen in a pharmaceutically acceptable carrier.

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14. A composition according to Claim 1 in the form of a kit comprising (i) an adjuvant component comprising the 1H-imidazo[4,5-c]quinolin-4-amine, and (ii) an immunogen component separate from the adjuvant 10 component and comprising the immunogen.

15. A method of increasing the immune response to an immunogen, comprising the step of administering (i) the immunogen in an amount effective to stimulate an 15 immune response, and (ii) as a vaccine adjuvant a 1H-imidazo[4,5-c]quinolin-4-amine in an amount effective to increase the immune response.

16. A method of increasing the immune response of 20 a mammal to an immunogen, comprising the step of administering to the mammal (i) the immunogen in an amount effective to stimulate an immune response, and (ii) as a vaccine adjuvant a 1H-imidazo[4,5-c]quinolin-4-amine in an amount effective to increase the immune 25 response.

17. A method of increasing the immune response of a fowl to an immunogen, comprising the step of administering to the fowl (i) the immunogen in an 30 amount effective to stimulate an immune response, and (ii) as a vaccine adjuvant a 1H-imidazo[4,5-c]quinolin-4-amine in an amount effective to increase the immune response.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/03295

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.C1. 5 A61K39/39

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
Int.C1. 5	A61K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X,P	THE JOURNAL OF INFECTIOUS DISEASES vol. 167, no. 3, March 1993, pages 731 - 735 DAVID I. BERNSTEIN ET AL. 'ADJUVANT EFFECTS OF IMIQUIMOD ON A HERPES SIMPLEX VIRUS TYPE 2 GLYCOPROTEIN VACCINE IN GUINEA PIGS.' THE WHOLE ARTICLE ---	1-17
Y	ANTIVIRAL RESEARCH vol. 10, 1988, pages 209 - 223 C.J. HARRISON ET AL. THE WHOLE ARTICLE ---	1-7, 11, 13, 14 -/-

¹⁰ Special categories of cited documents :¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

09 AUGUST 1993

Date of Mailing of this International Search Report

24 -08- 1993

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

REMPP G.L.E.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	ANTIMICROBIAL AGENTS AND CHEMOTHERAPY vol. 33, no. 9, September 1989, pages 1511 - 1515 DAVID I. BERNSTEIN ET AL. THE WHOLE ARTICLE ---	1-7, 11, 13, 14
Y	EP,A,0 389 302 (RIKER LABORATORIES, INC.) 26 September 1990 cited in the application see page 8, line 57 - page 10, line 31 ---	1-17
Y	EP,A,0 145 340 (RIKER LABORATORIES, INC.) 19 June 1985 cited in the application see page 15, line 28 - page 18, line 20 ---	1-17
Y	EP,A,0 385 630 (RIKER LABORATORIES) 5 September 1990 cited in the application see page 8, line 23 - page 10, line 13 -----	1-17

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/03295

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 15-17 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9303295
SA 73017

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

09/08/93

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		JP-A-	3027380	05-02-91